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Sulfite-oxido-reductase is involved in the oxidation of sulfite in *Desulfocapsa sulfoexigens* during disproportionation of thiosulfate and elemental sulfur

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Abstract

The enzymatic pathways of elemental sulfur and thiosulfate disproportionation were investigated using cell-free extract of *Desulfocapsa sulfoexigens*. Sulfite was observed to be an intermediate in the metabolism of both compounds. Two distinct pathways for the oxidation of sulfite have been identified. One pathway involves APS reductase and ATP sulfurylase and can be described as the reversion of the initial steps of the dissimilatory sulfate reduction pathway. The second pathway is the direct oxidation of sulfite to sulfate by sulfite oxidoreductase. This enzyme has not been reported from sulfate reducers before. Thiosulfate reductase, which cleaves thiosulfate into sulfite and sulfide, was only present in cell-free extract from thiosulfate disproportionating cultures. We propose that this enzyme catalyzes the first step in thiosulfate disproportionation. The initial step in sulfur disproportionation was not identified. Dissimilatory sulfite reductase was present in sulfur and thiosulfate disproportionating cultures. The metabolic function of this enzyme in relation to elemental sulfur or thiosulfate disproportionation was not identified. The presence of the uncouplers HQNO and CCCP in growing cultures had negative effects on both thiosulfate and sulfur disproportionation. CCCP totally inhibited sulfur disproportionation and reduced thiosulfate disproportionation by 80% compared to an unamended control. HQNO reduced thiosulfate disproportionation by 80% and sulfur disproportionation by 90%.

Abbreviations: CCCP – Carbonylcyanide m-chlorophenylhydrazone; HQNO – 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; APS – adenosine 5-phosphosulfate APAT – adenylylsulfate:phosphate adenylyltransferase

Introduction

Bacterial growth by disproportionation of inorganic sulfur compounds such as thiosulfate and sulfite was discovered by Bak & Cypionka (1987) e.g. $S_2O_3^{2-} + H_2O \rightarrow SO_4^{2-} + HS^- + H^+$. Since then, this process has been reported to take place in a number of sulfate-reducing bacteria (Bak & Pfennig 1987; Krämer & Cypionka 1989). Thamdrup et al. (1993) showed that disproportionation of elemental sulfur could sustain growth as well, $3\ S^\circ + 2\ FeOOH \rightarrow SO_4^{2-} + 2\ FeS + 2\ H^+$. This process requires that the sulfide concentration is kept low to be energetically favorable, however. Usually ferric iron is added to remove the sulfide that is produced. In general, microorganisms that disproportionate inorganic sulfur compounds are also able to grow by dissimilatory sulfate reduction. However, *Desulfocapsa sulfoexigens*, a marine bacterium closely related to the sulfate reducer and disproportionator *Desulfocapsa thiozymogenes*, is unable to reduce sulfate (Finster et al. 1998).

To this date, only few studies have addressed the pathway of the disproportionation of inorganic sulfur compounds (Böttcher et al. 2001; Cypionka et al. 1998; Füseler & Cypionka 1995; Füseler et al. 1996; Krämer & Cypionka 1989). Krämer & Cypi-

onka (1989) proposed that the first step of thiosulfate disproportionation is the reduction of thiosulfate by thiosulfate reductase. During this reaction sulfide and sulfite are formed. They found evidence for a reverse APS pathway by which sulfite is oxidized to sulfate. In more recent studies, Füseler et al. (1995, 1996) concluded that the aerobic oxidation of sulfide by sulfate-reducing bacteria proceeded via a pathway that produced elemental sulfur as an intermediate.

While few studies have addressed disproportionation pathways of inorganic sulfur compounds, numerous studies have investigated the aerobic as well as the anaerobic oxidation of reduced inorganic sulfur compounds (for review, see Brune 1989; Friedrich 1998; Friedrich et al. 2001; Kelly 1999; Kelly et al. 1997; Suzuki 1999). The disproportionation pathways outlined above and the pathways of sulfur-oxidation exhibit several similarities. Sulfite was identified as the key intermediate in the oxidation of sulfur compounds to sulfate (Kappler & Dahl 2001).

The oxidation of sulfite may occur along two different pathways (for review, see Kappler & Dahl 2001). The indirect pathway, known as the APS pathway, is a reversion of the sulfate reduction pathway in which the enzymes responsible for dissimilatory sulfate reduction operate in the reverse direction. In this case, ATP is generated through substrate level phosphorylation. Additional energy can be conserved via electron transport phosphorylation. The alternative enzymatic pathway involves the oxidation of sulfite by sulfite:oxidoreductase. This enzyme was found in a number of phylogenetic lineages such as thiobacilli and anoxygenic phototrophs (Kappler & Dahl 2001), but has not been described in sulfate reducers. Oxidation of sulfite by sulfite:acceptor oxidoreductase generates electrons with high reducing power (−516 mV) and is proposed to be involved in proton translocation.

In this communication, we present a study of the pathways of elemental sulfur and thiosulfate disproportionation in *Desulfocapsa sulfoexigens*. This organism was originally isolated from marine surface sediment of an inter-tidal mudflat in Arcachon Bay (France) (Finster et al. 1998) under anoxic conditions with hydrophilic elemental sulfur as the only energy source, bicarbonate as the only carbon source and ferric iron to keep the concentration of free sulfide low. It was shown that elemental sulfur was disproportionated into sulfide and sulfate at a 3:1 ratio. In addition to elemental sulfur, thiosulfate and sulfite sustained growth of *D. sulfoexigens* cultures by disproportionation. We have measured the activity of a number of enzymes, allowing us to reconstruct the entire pathway of thiosulfate disproportionation and parts of the pathway of elemental sulfur disproportionation. In addition, we have evidence of the presence of two pathways of sulfite oxidation in *D. sulfoexigens* that may function simultaneously.

Materials and methods

Organism and cultivation

Desulfocapsa sulfoexigens was obtained from our own culture collection. It was cultured under anoxic conditions with either thiosulfate or hydrophilic elemental sulfur as the only energy source in an anaerobic system consisting of a glass fermentor with a working volume of 2.5 liters. The culture was stirred with a Teflon-coated magnet at 300 rpm. The medium composition was as described by Finster et al. (1998). Acetate (final concentration 2 mM) was added as a carbon source. The pH was kept within the interval of 6.7–7.0 by automatic addition of NaOH and HCl from 0.1 M stock solutions. The fermentor was incubated in a thermostated water bath (30 °C). Sulfide was removed by continuously flushing the culture with a gas mixture of 10% CO_2 and 90% N_2. H_2S was trapped in a solution of ZnAc (20% w/vol) as ZnS.

Preparation of cell-free extracts

The bacteria were harvested by centrifugation for 30 min at 15000 g and washed twice in a buffer solution containing 100 mM Tris-HCl pH 7.5; 2% NaCl; 2 mM $MgCl_2$; 2 mM dithiothreitol. The buffer solution was flushed with N_2 before use. Cells were stored at −20 °C or −80 °C.

The cell suspensions were thawed and portions of 1–5 ml were taken for cell extract preparation by sonication. Before sonication, N_2/CO_2-flushed milli-Q water was added to the cell suspension (2 vol. suspension + 1 vol. H_2O) to lower the salinity and induce cell breakage. This procedure also serves to facilitate handling of the cell suspension during sonication and the subsequent microscopical examination. The cells were sonicated on ice under a N_2-atmosphere for 3 times 20 seconds. After sonication, the effect of the treatment was examined microscopically, and the treatment was repeated if a large fraction of cells was still intact. Afterwards, the suspension was centrifuged to remove the cell debris. The cell-free extract (the supernatant) was transferred to a N_2/CO_2-flushed

test tube that was sealed with a rubber septum and kept on ice during the subsequent enzyme assays, after which it was stored at −20 °C. The cell-free extracts obtained from the different cultures varied in protein content.

With thiosulfate the protein content of the cell-free extract varied between 3.200 and 6.940 mg ml^{-1}, with elemental sulfur the protein content varied between 0.490 and 0.930 mg ml^{-1}. The difference in protein content was probably due to variable efficiency of the sonication procedure. The presence of elemental sulfur in the cell pellet may have impeded sonication due to unspecific absorption (Phillips 1994).

Determination of enzyme activities

Unless otherwise specified, the enzyme assays were performed in N_2-flushed solutions in a quartz cuvette sealed with a butyl rubber stopper. Additions were made with microliter syringes. Background activities of the extracts in the reaction mixture were determined as a control before addition of the initiating reagent (differs from assay to assay, see references for further details). Activity is defined as detectable difference in absorbance between the background activity and the initiated activity. Data were recorded on a personal computer with a temporal resolution of 0.1 sec. The reactions catalyzed by the enzymes are depicted in Table 1.

ATP-sulfurylase (EC 2.7.7.4) was assayed according to the method described by Krämer & Cypionka (1989) at pH 7.5 and additionally as described by Dahl & Trüper (1994) at pH 8.0. No difference was observed. The activity was determined by measuring the adenosine-5′-phosphosulfate (APS) and pyrophosphate-dependent production of ATP in a coupled photometric assay. The ATP production was followed via the phosphorylation of glucose (hexokinase EC 2.7.1.1) and the subsequent oxidation to 6-phosphogluconolactone by $NADP^+$ (glucose-6-phosphate dehydrogenase EC 1.1.1.49) (Groenestijn et al. 1987; Guillory & Fisher 1972). Reduction of $NADP^+$ was measured by following the increase in absorbance at 340 nm (ϵ_{340} = 6100 cm^{-1} M^{-1}). The obtained values were corrected for chemical reduction of $NADP^+$, which was recorded before addition of the extract.

Pyrophosphatase activity is known to interfere with this assay, because pyrophosphate serves as substrate for both enzymes (Dahl & Trüper 1994). *Adenylylsulfate:phosphate adenylyltransferase APAT (formerly ADP-sulfurylase* (EC 2.7.7.5) (Bruser et al. 2000)) and Adenylate kinase (EC 2.7.4.3) measurements were based on the coupled assay used for ATP sulfurylase, although modified according to Krämer & Cypionka (1989).

Hydrogenase (EC 1.18.99.1) was assayed by measuring the reduction of methylviologen with hydrogen under strict anaerobic conditions according to Badziong & Thauer (1980).

APS reductase (EC 1.8.99.2) was assayed by measuring the AMP-dependent reduction of ferricyanide (420 = 1090 cm^{-1} M^{-1}) with sulfite according to Krämer & Cypionka (1989). To test the consistency of the specific activities obtained under different reaction conditions (such as pH, initiating reagent and reactant concentrations, see references for further details), additional tests were carried out under other reaction conditions as described by Lampreia et al. (1994) and Dahl & Trüper (1994). The method of Krämer & Cypionka (1989) resulted in the highest APS reductase activities recorded in this study. The extract was allowed to incubate in the mixture for 1 minute before addition of sulfite. The reaction was initiated by adding AMP after the background rate of ferricyanide reduction had been established. The reduction of ferricyanide was monitored by following the decrease in absorbance at 420 nm.

Sulfite oxidoreductase (EC 1.8.2.1) was assayed by the ferricyanide-dependent continuous assay described by Dahl & Trüper (1994) at pH 9 and also by the procedure described by Krämer & Cypionka (1989) at pH 8. No significant difference was observed. The reaction was initiated by addition of cell-free extract after the background rate of ferricyanide autoreduction was established. Furthermore, any endogenous reduction of ferricyanide by the enzyme preparation (without sulfite) was assayed as a control. The reduction of ferricyanide was monitored by following the decrease in absorbance at 420 nm. Two mole of ferricyanide oxidize one mol of sulfite.

Sulfite reductase (EC 1.8.99.3) activity was determined in a continuous spectrophotometric assay conducted under strictly anoxic conditions as described by Dahl et al. (1994). The activity was recorded by following the decrease in absorbance of reduced methyl viologen at 600 nm (ϵ_{600} = 13000 cm^{-1} M^{-1}). Methyl viologen was chemically reduced by addition of small amounts of dithionite to the N_2-flushed stock solution.

Thiosulfate reductase (EC 2.8.1.3) was assayed by following the oxidation of reduced methylviologen

Table 1. Enzyme activities demonstrated in cell-free extracts of *Desulfocapsa sulfoexigens* cultivated under different growth conditions. Values in μmol substrate min^{-1} mg $protein^{-1}$. (–) Activity not detected. The rate of thiosulfate consumption in the thiosulfate disproportionating culture was 0.1 μmol min^{-1} (mg protein)$^{-1}$ and the rates of sulfate and sulfide production in the elemental sulfur disproportionating culture were 0.046 μmol min^{-1} (mg protein) $^{-1}$ and 0.013 μmol min^{-1} (mg protein) $^{-1}$, respectively. Rates of the specific enzyme activities that were high enough to account for the consumption rate of thiosulfate and production rates of sulfide and sulfate by intact cells are written in bold. The sign > is used because formation of orthophosphate was very rapid and could not be followed continuously. To all cultures acetate was added as a carbon source

Enzyme	Reaction catalyzed by the enzyme	Extract obtained from culture of *D. sulfoexigens* grown with:		
		Thiosulfate	Sublimed elemental sulfur	Sublimed elemental sulfur uncoupler HQNO
Adenylate kinase	$2\ ADP \rightarrow ATP + AMP$	**0.103**	0.011	0.018
APATadenylylsulfate:phosphate adenylyltransferase	$APS + P_i \rightarrow SO_4^{2-} + 2H^+ + ADP$	–	–	0.004
APS reductase	$HSO_3^- + AMP + H^+ \rightarrow APS + H_2$	**3.570**	**0.228**	**0.096**
ATP sulfurylase	$APS + PP_i \rightarrow SO_4^{2-} + 2H^+ + ATP$	0.012	0.021	–
Hydrogenase	$H_2 \rightarrow 2H^+ + 2\ e^-$	0.075	–	–
Pyrophosphatase	$PP_i + H_2O \rightarrow 2\ P_i$	>**0.410**	>**0.430**	>**0.160**
Sulfite oxidoreductase	$SO_3^{2-} + H_2O + [H]^+ \rightarrow SO_4^{2-} + [H]$	**0.256**	**0.348**	**0.027**
Sulfite reductase	$HSO_3^- + 3\ H_2 \rightarrow HS^- + 3\ H_2O$	0.023	0.005	0.004
Thiosulfate reductase	$S_2O_3^{2-} + H_2 \rightarrow HSO_3^- + HS^-$	**0.181**	–	–

(ϵ_{578} = 9600 cm^{-1} M^{-1}) with thiosulfate. The assay was conducted according to Krämer & Cypionka (1989) with one small modification. Methylviologen was reduced by addition of small amounts of dithionite to the N2 flushed solution instead of reduction by HCl-washed zinc granules.

Pyrophosphatase (EC 3.6.1.1) was assayed by discontinuously measuring the formation of orthophosphate from pyrophosphate (Chen et al. 1956) under anoxic conditions. The test was performed in 5-ml stoppered tubes as described by Krämer & Cypionka (1989). The formation of orthophosphate was very rapid and could not be followed continuously. The calculations of the specific activities were biased by a time-consuming sampling procedure. Sampling was possible approximately once every 30 seconds, and this may have led to underestimation of the activity.

Thiosulfate dehydrogenase (EC 1.8.2.2) was assayed according to Visser et al. (1996). The extract was allowed to incubate for 1 min before addition of thiosulfate to induce the reaction.

Rhodanese (EC 2.8.1.1) was assayed as described in Kelly & Wood (1994) by discontinuous determination of the thiocyanate product, based on the method of Sörbo (1953). The potential supply of reactive substances from the extract was determined by addition of cell-free extract without addition of thiosulfate.

Polysulfide reductase (EC 1.97.1.3) was determined by recording the reduction of polysulfide (ϵ_{360} = 36000 cm^{-1} M^{-1}) with hydrogen at 360 nm under anoxic conditions (Fauque et al. 1994).

Sulfur oxidoreductase (EC 1.97.1.3) activity was determined by modification of the colorimetric assay described by Fauque et al. (1994), originally developed by Zöphel et al. (1988). Phenosafranin was reduced chemically by NADH and small amounts of dithionite. The reaction was conducted under strictly anoxic conditions in a N_2 atmosphere in a cuvette sealed with a butyl rubber stopper, through which the reagents and the extract were supplied with microliter syringes. The oxidation of phenosafranin was measured as an increase in absorbance at 525 nm.

Inhibitor studies

The effect of inhibitors on disproportionation of thiosulfate and elemental sulfur and culture growth was studied after addition of either 50 μM of the uncoupler CCCP and 20 μM of the electron transport inhibitor HQNO to growing batch cultures. The inhibitor stock solutions were freshly prepared in small volumes of 70% ethanol. Experiments were carried out in batch cultures as described by Finster et al. (1998). In one run of the fermentor HQNO (final concentration 20 μM) was added to a culture grown with elemental sulfur during the linear growth phase.

Analytical procedures

Total protein in the cell-free extracts was determined by the micro-burette method of Goa (Brewer et al. 1977) based on the chemical determination of peptides using a $CuSO_4$ reagent. Measurements were conducted at two different wavelengths (330 and 540 nm). Bovine serum albumin was used as standard. Autoabsorption of the extracts was determined prior to addition of the reagent. Sulfide was determined spectrophotometrically according to Cline (1969). Sulfate and thiosulfate concentrations were quantified by suppressed ion chromatography (Finster et al. 1998). Sulfite was not determined by ion chromatography because it was rapidly oxidized during the analytical procedure.

Sulfite was instead determined spectrophotometrically by use of fuchsin reagent (Pachmayr 1960: Bak & Pfennig 1987). The analysis of sulfite was conducted in 10-ml volumetric bottles. Thiosulfate and sulfide interfere with this sulfite assay. In thiosulfate disproportionating cultures the sulfite determination was only reliable after the thiosulfate pool was exhausted. It is not thiosulfate as such that reacts with the fuchsin reagent but sulfite that is produced from the chemical disproportionation of thiosulfate in acidic solution. Sulfide interference, which was much weaker, could be corrected for by calibration with sulfide standards. There was no interference with elemental sulfur.

Cell counts were performed on formaldehyde preserved samples. Cells were counted after staining with acridine orange as described by Finster et al. (1998).

Chemicals

All chemicals were obtained from commercial suppliers.

Results

The sulfide and sulfate production rates in thiosulfate disproportionating cultures at time of cell harvest were 0.09 and 0.08 μmol min^{-1} (mg protein) $^{-1}$, respectively. The thiosulfate consumption rate was 0.10 μmol min^{-1} and (mg protein) $^{-1}$. The sulfide and sulfate production rates in elemental sulfur disproportionating culture were 0.046 and 0.013 μmol min^{-1} (mg protein) $^{-1}$, respectively. Sulfite concentrations between 10 and 30 μM were determined during growth of *D. sulfoexigens* on elemental sulfur and after thiosulfate was consumed. Cell densities of 1.9×10^9 and 0.6×10^9 cells $\times$ ml^{-1} were obtained on thiosulfate and elemental sulfur, respectively. After HQNO addition the culture grew very slowly and reached a final density of 0.1×10^9 cells $\times$ ml^{-1}.

Enzyme assays

Activities of the following enzymes could not be detected in any of the cultures: thiosulfate dehydrogenase, polysulfide reductase, sulfur oxidoreductase and rhodanese. The enzyme activities are listed in Table 1.

Enzyme activities in cell-free extract of the thiosulfate disproportionating cultures: The activity of thiosulfate reductase was measured in the extract obtained from thiosulfate disproportionating cultures. The activity of this enzyme was high enough to account for the formation of sulfide and for the consumption of thiosulfate in agreement with the reaction given in Table 1. Thiosulfate is disproportionated into sulfide and sulfate at a 1:1 ratio. Another enzyme that may catalyze the production of sulfide is the dissimilatory sulfite reductase (DSR). Although active in the culture, the activity of this enzyme was too low to account for the calculated rate of sulfide formation.

On the oxidative side, the activity of sulfite oxidoreductase was demonstrated. The activity of this enzyme was high enough to account for the sulfate formation rate obtained in the culture. In addition, APS reductase and ATP sulfurylase activities were measured. The activity of APS reductase was sufficiently high to account for the formation of sulfate. The activity of ATP sulfurylase, which in a reverse reaction catalyzes the formation of sulfate and ATP from APS, was about 20 times too low to account for the formation of sulfate in the culture. The measured pyrophosphatase activity was high enough to supply the ATP sulfurylase with pyrophosphate and to account for the formation of sulfate and ATP. Adenylate kinase and hydrogenase activity was present.

Enzyme activities in cell-free extracts from elemental sulfur disproportionating cultures: In contrast to the thiosulfate pathway, the pathway of elemental sulfur disproportionation was only partly resolved. No thiosulfate reductase activity was found in extracts from cultures grown with elemental sulfur. However, the activities of all the other enzymes present in thiosulfate cultures were also measured in cultures grown with elemental sulfur. The activity of sulfite oxidoreductase was high enough to account for the production of sulfate in the culture. The activities of

APS reductase and ATP sulfurylase were sufficiently high to explain the formation of sulfate in the culture grown with elemental sulfur. Yet the activity of ATP sulfurylase is only 10% of the activity of APS reductase. The measured activity of pyrophosphatase was high enough to supply the ATP sulfurylase with pyrophosphate and to account for formation of sulfate and ATP. The activity of sulfite reductase was too low to account for the sulfide production observed in the culture. This indicates that sulfide may have been produced by another (or an additional) enzymatic reaction. Adenylate kinase activity was present.

Effect of inhibitors

The presence of CCCP completely inhibited growth on elemental sulfur, whereas the growth rate on thiosulfate decreased by 80%. HQNO reduced the growth rate by 95% on sulfur and 80% on thiosulfate. The ratios of sulfide:sulfate were not affected by the presence of the inhibitors. The addition of HQNO to an elemental sulfur disproportionating fermentor culture did not only reduce the growth rate and sulfate and sulfide production by 90% compared to an unamended culture but also decreased activities of several enzymes. (Table 1). ATP sulfurylase was no longer detectable. APS reductase and pyrophosphatase activities decreased by approximately 60%. Sulfite oxidoreductase activity decreased by about 90%. Sulfite reductase activity was almost unchanged, while adenylate kinase activity was stimulated by 60%. In contrast to incubations without HQNO, the activity of adenylylsulfate:phosphate adenylyltransferase (APAT) was measured.

Discussion

Our study demonstrates the presence of a number of enzymes that are involved in the disproportionation of thiosulfate and elemental sulfur by *D. sulfoexigens*. The results of the enzyme studies as well as the presence of sulfite in the culture medium point to sulfite as an important intermediate in both thiosulfate and elemental sulfur disproportionation. In both cases, sulfite may be oxidized directly to sulfate by sulfite oxidoreductase (II), or the oxidation may proceed via APS formation involving APS reductase (III), pyrophosphatase and ATP sulfurylase (IV) (Figure 1). Interestingly, formation of ADP via APAT (V) also seems to be possible. The formation of sulfite, however, seems to proceed via different pathways in thiosulfate and elemental sulfur disproportionating cultures, respectively (Figure 1). In thiosulfate disproportionating cultures sulfite is produced by thiosulfate reductase (I). The oxidation of sulfur to sulfite (VIIa and VIIb) still needs to be resolved. Generally, the activation and conversion of elemental sulfur is a persistent problem in research fields involving this compound. Recent studies (Cypionka et al. 1998; Thamdrup et al. 1993; Füseler & Cypionka 1995; Füseler et al. 1996; Böttcher et al. 2001) have suggested that sulfite is an intermediate in both the disproportionation and the oxidation of elemental sulfur. Cypionka et al. (1998) proposed elemental sulfur as an intermediate in thiosulfate disproportionation and Thamdrup et al. (1993) suggested that thiosulfate is an intermediate in S^0 disproportionation. Enzymatic studies of the pathway of S^0 disproportionation have not hitherto been performed. However, previous culture studies proposed that sulfite is an intermediate in the disproportionation of elemental sulfur produced during sulfide oxidation in cultures of *Desulfobulbus propionicus* and *Desulfovibrio desulfuricans* (Füseler & Cypionka 1995; Füseler et al. 1996). The authors did not propose a pathway that might lead to the formation of sulfite. A recent study on sulfur isotope fractionation during disproportionation of elemental sulfur by *Desulfocapsa thiozymogenes* also suggested that sulfite is a likely intermediate in this process (Böttcher et al. 2001). Our results are in good agreement with these findings.

Enzymology of elemental sulfur conversion

The oxidative metabolism of elemental sulfur and other inorganic sulfur compounds has recently been reviewed (Friedrich 1998; Kelly 1999; Kelly et al. 1997; Suzuki 1999). Generally, the authors concluded that the central problem is the enzymology of the conversion of elemental sulfur to sulfate and/or sulfite in bacteria, especially in the absence of molecular oxygen. Some mechanisms have been proposed, however, and the role of sulfite reductase, an enzyme that has been shown to catalyze the conversion of other substrates than sulfite, has been particularly stressed. When acting in the reverse direction, sulfite reductase (VI) oxidizes sulfide, polysulfide and elemental sulfur to sulfite. Evidence of this mechanism has been found in for instance *Thiobacillus denitrificans* (Kelly 1999; Hipp et al. 1997; Trüper 1994) and in *Allochromatium* (formerly *Chromatium*) *vinosum* (Schedel et al. 1979).

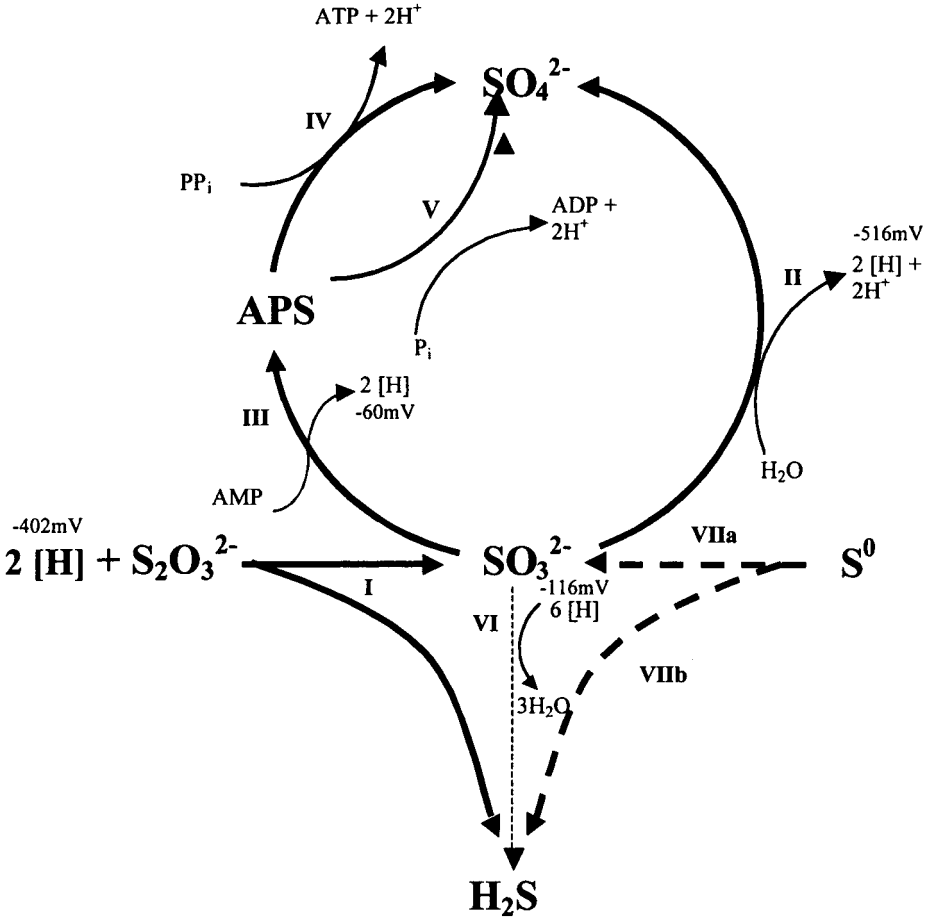

Figure 1. The proposed pathways of thiosulfate and elemental sulfur disproportionation by *D. sulfoexigens*. I. Thiosulfate reductase. II: Sulfite oxidoreductase. III: APS reductase. IV: ATP sulfurylase. V: APAT. VI: Sulfite reductase. VIIa and VIIb: Unresolved reactions that may proceed via unidentified intermediates. Full bold lines indicate the reactions proposed to occur on the basis of positive enzyme assays. Dotted thin line (i.e. reaction VI) indicates that *D. sulfoexigens* possesses the enzyme responsible for the reaction, but that it is not thought to be involved in the process of disproportionation. Dotted lines indicate that activity of the enzyme responsible for the reaction was not detected in the culture.

Sulfite reductase found in *D. sulfoexigens* may function in a similar way when elemental sulfur serves as substrate for this enzyme. The presence of sulfite reductase in cells of *D. sulfoexigens* was supported by other observations: 1) The capability of *D. sulfoexigens* to reduce sulfite to sulfide in the presence of hydrogen (Frederiksen & Finster, sub.), and 2) Sequencing of the DSR gene from a culture of *D. sulfoexigens* (T.R. Thomsen, unpublished data).

Presence of sulfite oxidoreductase in Desulfocapsa sulfoexigens

Our finding that in *Desulfocapsa sulfoexigens* sulfite oxidoreductase (II) may oxidize sulfite directly to sulfate is very interesting. To our knowledge, this enzyme has not been demonstrated in delta-proteobacterial sulfate reducers, to which *D. sulfoexigens* is closely related. The only study in which this enzyme was assayed was carried out on *D. sulfodismutans* by Krämer & Cypionka (1989) with negative results. However, in the discussion the authors point out the advantage this enzyme would give if active during thiosulfate disproportionation. Sulfite oxidoreductase (II) generates electrons at a very negative reductive potential (−516 mV) that could be used in the initial step of thiosulfate disproportionation.

Sulfite oxidoreductase (II) is well known in phototrophic and chemotrophic sulfur oxidizing bacteria. Interestingly, many of these bacteria are also capable of oxidizing sulfite by the APS reductase (III and VI) pathway. The simultaneous presence of both sulfite oxidation pathways has been established for a num-

ber of chemo- and photolithotrophic sulfur oxidizers from the β- and γ-Proteobacteria, the green sulfur bacteria, the Gram-positive bacteria and the archaea (Kappler & Dahl 2001). A study by Sanchez et al. (2001) examined the *in vivo* role of the two pathways in *Allochromatium vinosum*. This study indicates that the presence of the second pathway (sulfite oxidoreductase (II)) allowed a higher rate of supply of reducing power. In addition, this pathway does not require pyrophosphate. The *in vivo* role of these two sulfite-oxidizing pathways in *Desulfocapsa sulfoexigens* was not investigated in this study. Other studies stress the operation of the inverse sulfate reduction pathway, however. *D. sulfoexigens* possesses the enzymes responsible for dissimilatory sulfate reduction, but it has not been possible to grow this organism as a sulfate reducer (Finster et al. 1998; unpublished data). It has been shown, however, that molybdate inhibits the disproportionation of elemental sulfur and thiosulfate, and Finster et al. (1998) proposed that molybdate interfered with the formation of APS. This points to an operational reverse sulfate reduction pathway, which is inhibited by molybdate (Newport & Nedwell 1988). Sulfate is known to inhibit sulfite oxidoreductase (II) by product inhibition (Quentmeier et al. 2000; Suzuki 1994), and the sulfate analogue molybdate has the same effect. This may present a plausible explanation for the fact that molybdate completely inhibits sulfur disproportionation, despite the potential operation of two distinct pathways of sulfite oxidation. In addition, the operational reverse APS pathway may deplete the cell for ATP in the presence of molybdate despite the function of the sulfite oxidoreductase pathway.

The effect of inhibitors on the disproportionation of elemental sulfur and thiosulfate

It has recently been shown (Masau et al. 2001), that sulfite oxidation is inhibited by HQNO and the uncoupler CCCP (Krämer & Cypionka 1989: Meulenberg et al. 1992; Beffa et al. 1993). Meulenberg et al. (1992) furthermore showed that CCCP partly inhibits the respiratory oxidation of thiosulfate in sulfur oxidizing bacteria of the genus *Thiobacillus*, and that the oxidation of elemental sulfur was completely inhibited. Two reasons were given to explain this observation. An energized membrane is required for activation of the chemically inert S_8 ring structure, or alternatively the uncoupler sensivity might reflect the involvement of active transport mechanisms.

In this study, thiosulfate disproportionation was inhibited by 80% (by growth rate) by CCCP as well as by HQNO compared to an inhibitor free culture. Most likely, these compounds inhibited sulfite oxidation. Because reducing equivalents from sulfite oxidation are needed to disproportionate thiosulfate to sulfide and sulfite (I), the presence of either one of these inhibiting compounds would result in a decrease of both reactions. The unchanged ratio of produced sulfide:sulfate (3:1) supports this idea. The total inhibition of sulfur disproportionation by the uncoupler CCCP suggested that this compound did not inhibit sulfite oxidation alone but affected an active transport step, which might be involved in the oxidation of S^0 or in its activation as it previously has been suggested for *Thiobacillus ferrooxidans* (Hazeu et al. 1988) and for *Thiobacillus acidophilus* (Meulenberg et al. 1992). The state of the membrane potential could play an essential role during S^0-activation, -reduction or -oxidation. This idea is supported by Beffa et al. (1993) who concluded that the proton potential could be the direct energy donor for the activation of the S^0 molecule in *Thiobacillus denitrificans*.

The effect of CCCP on thiosulfate disproportionation differs between the two *Desulfovibrio* strains *Desulfovibrio sulfodismutans* and *Desulfovibrio desulfuricans* on the one hand side and *Desulfocapsa sulfuexigens* on the other hand side. Krämer & Cypionka (1989) interpreted the total inhibition of thiosulfate disproportionation by CCCP in support of the absence of the sulfite oxidoreductase pathway, in addition to the fact that they were not able to detect the activity of this enzyme. Along with their line of arguments we interpret the partial inhibition of thiosulfate disproportionation in *D. sulfoexigens* by CCCP in support of the enzyme assay data, which clearly indicates the presence of this enzyme. Thus *D. sulfoexigens* saves energy, which otherwise would be required for the reverse electron transport to thiosulfate. The activity of both pathways allows *D. sulfoexigens* to generate ATP both by substrate level phosphorylation and proton motive force.

Conclusion

Our study has presented important findings that contribute to the elucidation of the enzymatic pathways involved in the disproportionation of thiosulfate and elemental sulfur. The initial step in the disproportionation of thiosulfate was mediated by thiosulfate

reductase (I), which produces sulfite and sulfide in a 1:1 ratio. The enzyme responsible for the initial conversion of elemental sulfur (VIIa and VIIb) has not been identified, but sulfite reductase (VI) may play a role in this process. Further studies are needed to explain this step in the pathway of elemental sulfur disproportionation. Sulfite is an essential intermediate in the disproportionation of both thiosulfate and elemental sulfur, and *D. sulfoexigens* is capable of oxidizing sulfite by two different pathways, the sulfite oxidoreductase (II) pathway and the APS reductase pathway (III) via ATP sulfurylase (IV) or APAT (V).

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